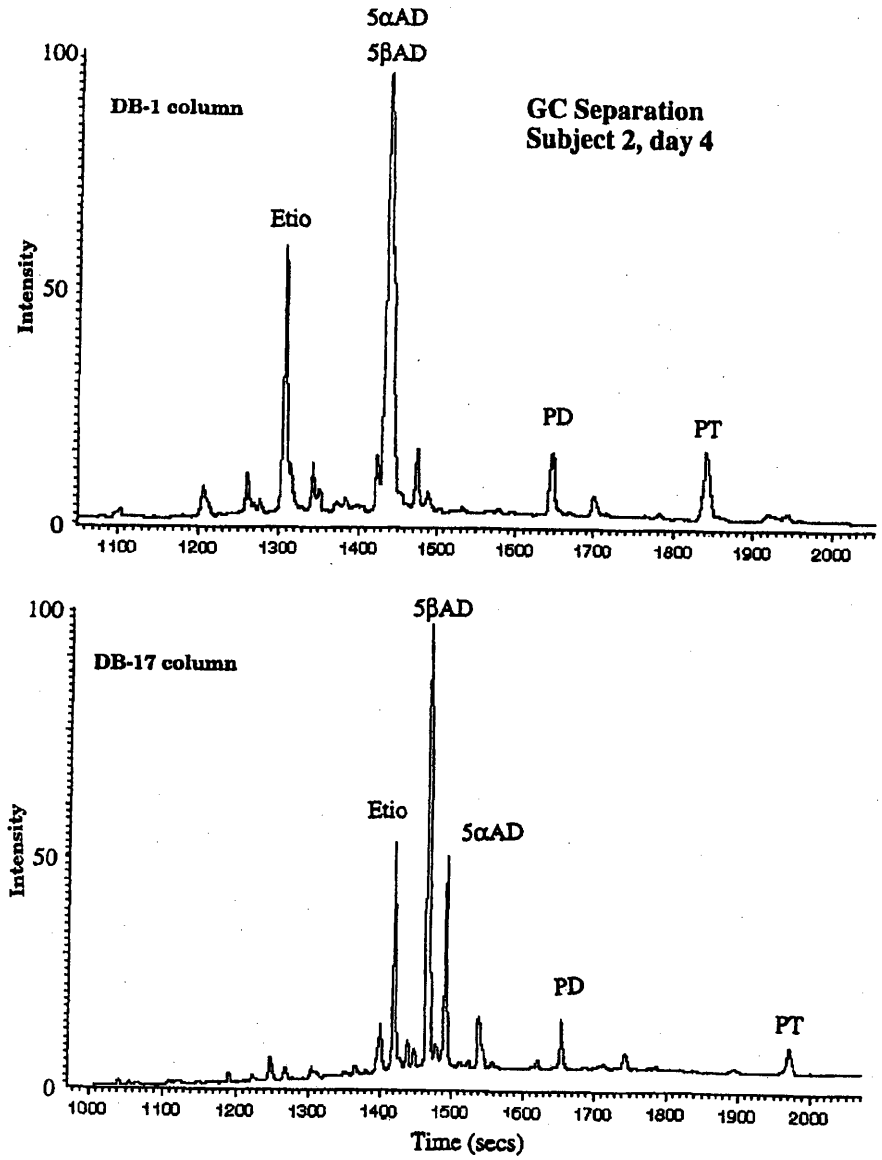

Figure 3 Gas chromatographic separation (on GC/C/IRMS instrument) of acetylated urinary steroid fraction. This sample was initially separated on a DB1 column, which failed to resolve the 5α- and 5β-androstanediols (upper panel). Later employment of a DB17 column permitted individual measurement of the epimers (lower panel). Both columns also resolved etiocholanolone, produced by bismuthate oxidation of pregnanetriol, pregnanediol, and nonoxidized pregnanetriol.

−29.28 and −29.15, respectively; and one Russian −30.22. The U.S. sample of more than 40 years old gave a value of −33.18, probably reflecting the different plant origin of synthetic steroids made long ago. We believe that testosterone is currently made mostly from soy by-products, although originally sterol constituents of the Mexican yam were the primary precursors.

Results for individual subjects

With the exception of Subject 1, a DB17 capillary column was utilized that allowed separate analysis of 5α- and 5-β androstanediols. Graphs illustrating the $\delta^{13}C^{0}/00$ results are shown in Figure 4A–F. In all cases, the $\delta^{13}C^{0}/00$ values for the androstanediols fell significantly following testosterone administration.

Figure 4 illustrates two other features. One is the adequacy of making single measurements of $\delta^{13}C^{0}/00$ for each sample. Panel B and C show results obtained by plotting the average of duplicate measurements (B) and of the *first* measurement of the duplicate pair (C). Essentially these graphs are identical, suggesting duplicate GC/C/IRMS analyses of the same preparative extract are unnecessary. The second feature relates to the trend of slightly increasing δ values for pregnanediol during the study period of Subject 3 (Figure 4D). Because this is not caused by random irreproducibility we proposed that the individual changed his diet substantially to foodstuffs with higher $\delta^{13}C^{0}/00$ values.

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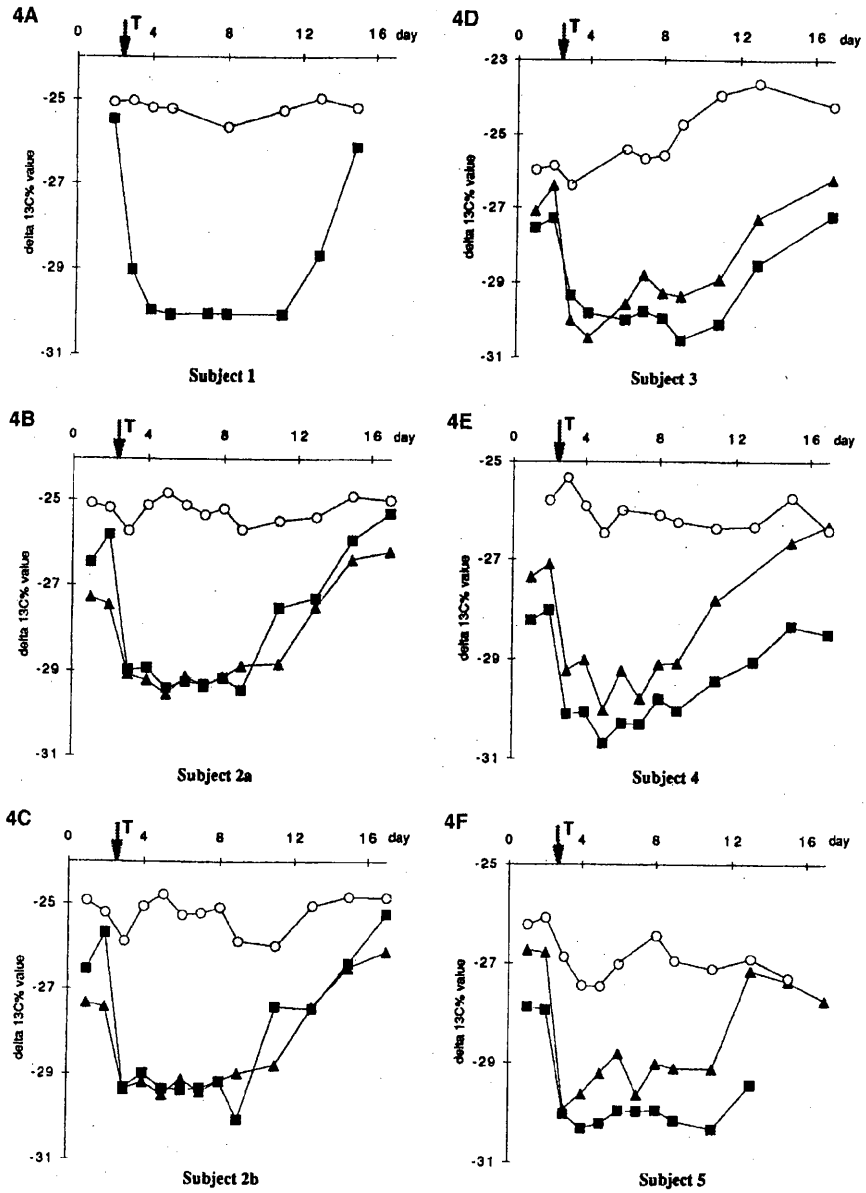

Figure 4 Values of $\delta^{13}C^{0}/00$ for all five subjects throughout the study period. The open circles represent pregnanediol, the closed rectangles 5βAD and closed triangles 5αAD. 4A shows the values obtained for Subject 1 in which only one baseline sample was analyzed, the testosterone being administered on the 2nd day of study. Both androstanediols were measured together on a DB1 column, 4B shows plots for Subject 2 obtained by averaging values for the duplicate runs, and 4C shows equivalent plots through only using the first analysis. 4C, 4D, and 4E are plots of Subjects 3, 4, and 5. For Subjects 2–5 the testosterone was administered after the second baseline sample (day 3 of study).

Designing a method for universal use demands final agreement of numerative values above and below which individuals are considered to have or to have not illicitly used a drug. In our studies with the Chinese subjects, it can be stated that for the five individuals, none had androstanediol $\delta^{13}C^{0}/00$ values less than −28.3 during the control period, and establishing a conservative cut-off value of −29.0 would clearly pick up all drug users for about 7 days after administration. Additionally, we considered that measurement of analyte/ECR ratios could allow numerative values to be established that are to some degree independent of variables introduced through using the methodology in different laboratories with different instrumentation. In Figure 5A, we show the $\delta^{13}C^{0}/00$ ratio of combined androstanediols (average of 5α- and 5β-) to pregnanediol (PD) for the five individuals studied so far. All baseline ratios fell below 1.08, and from these data, we would suggest that a ratio of greater than 1.1 could be used to confirm testosterone administration. When we averaged the data for the five individuals, we found that the AD/PD ratio value of 1.1 was exceeded for 11 days (Figure 5B). Because a combined androstanediol measurement is carried out in the Aguilera et al. studies,[5] comparable data could be prepared from their measurements, although in their case, cholesterol would be the ERC used for ratio determination.

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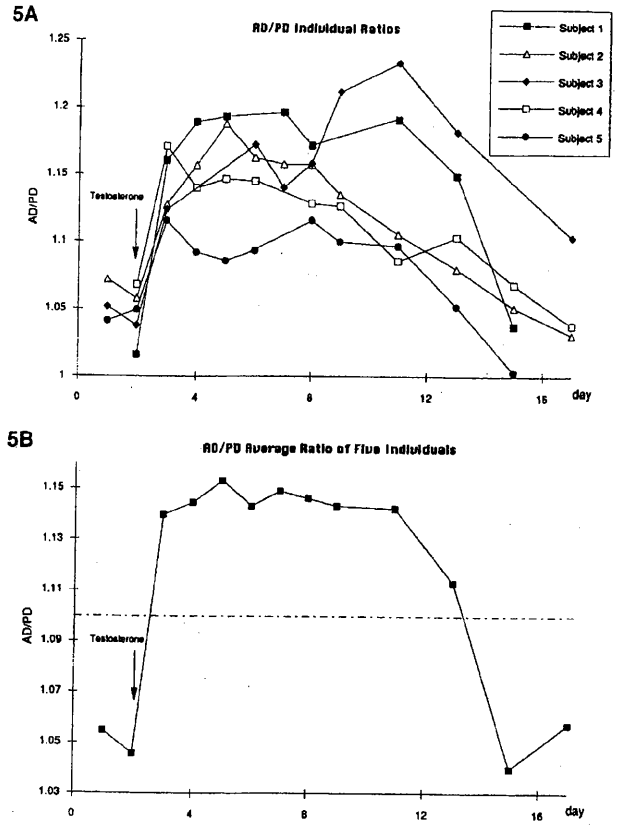

Figure 5 Androstanediol/pregnanediol ratio. A) ratios of $\delta^{13}C^0/00$ for the androstanediols (mean of 5α and β) to pregnanediol for the five subjects; B) average of the ratio for the five subjects for each day; the dotted line represents the proposed discriminant value (1.10) for confirmation of testosterone administration.

Reproducibility of isotope measurements and accuracy

For the five subjects studied, ideally 26 or more isotope measurements were made for each steroid; i.e., 13 duplicate analyses. A few samples were lost or contaminated, and occasionally only single measurements were made on a sample. As a representative analysis, Table 1 gives all of the measurements for Subject 2. Using these data, there are two features of accuracy and reproducibility that can be quantified. One is the variation in δ measurement between duplicate analyses of the same sample and the other the overall variation occurring during analyses of a complete series from one individual. For duplicates, the reproducibility was excellent and did not differ significantly between the five subjects studied. Considering the data shown in Table 1, averages and standard deviations were measured for each duplicate for the two androstanediol and pregnanediol diacetates that give values in the $\delta^{13}C^0/00$ −23 to −30 range. Means were then determined of the standard deviations. For pregnanediol, the mean standard deviation between duplicates was ± 0.21 with a range of ± 0.02 to ± 0.71. Comparable results were obtained for the androstanediols (± 0.11 for 5α-androstanediol and ±0.25 for 5β-androstanediol). These results were considered to represent excellent reproducibility and (as previously noted) suggested that the methodology could be used with *single* determinations by GC/C/IRMS of $\delta^{13}C^0/00$ for each steroid. This was illustrated graphically in Figure 4B and C, which showed both the $\delta^{13}C^0/00$ values for the average of duplicates and for the first analysis. The graphs were essentially identical.

Within a 17-day series of datapoints for duplicate assays for a single individual, the results show considerably less reproducibility—notice the peaks and valleys of the pregnanediol plots in Figure 4. In any physicochemical analyses, it is, of course, possible to have excellent reproducibility but compromised values. For accuracy and reproducibility assessment between a series of different samples, we use the δ measurement of the ERC pregnanediol. The administration of testosterone should in no way change the $\delta^{13}C^0/00$ value for pregnanediol that has to be produced from cholesterol via several intermediates. In a perfect analysis, the measured ^{13}C content of pregnanediol would not be expected to change during the 17-day study period unless, of course, the individual alters his or her diet markedly during the study period. Even with dietary modification, the ^{13}C content of body molecular constituents would be expected to change only slowly.

The highest and lowest duplicate average $\delta^{13}C^0/00$ values for pregnanediol for the five subjects differed by −0.92 to −2.96. As previously mentioned, with the exception of Subject 2, the average duplicate values seemed to rise and fall randomly during the 17-day study period. What

Table 1 $\delta^{13}C^0/_{00}$ values obtained from duplicate GC/C/IRMS analyses for Subject 2 before and following testosterone administration

	Subject 2		
	5βAD	5αAD	PD
Day 1	−26.55	−27.33	−24.93
	−26.15	−27.20	−25.34
	−26.66	−27.28	−25.15
Day 2	−25.68	−27.42	−25.20
	−25.95	−27.49	−25.17
Day 3[a]	−29.33	−29.36	−25.88
	−28.63	−28.81	−25.61
Day 4	−28.99	−29.19	−25.06
	−28.88	−29.24	−25.21
Day 5	−29.34	−29.49	−24.78
	−29.48	−29.61	−24.87
Day 6	−29.37	−29.11	−25.26
	−29.12	−29.16	−24.97
Day 7	−29.33	−29.42	−25.22
	−29.31	−29.28	−25.46
Day 8	−29.16	−29.16	−25.09
	−29.18	−29.14	−25.30
Day 9	−30.06	−28.98	−25.88
	−28.83	−28.78	−25.52
Day 11	−27.41	−28.78	−25.99
	−27.63	−28.88	−24.99
Day 13	−27.47	−27.42	−25.05
	−27.12	−27.59	−25.72
Day 15	−26.39	−26.50	−24.84
	−25.50	−26.28	−24.96
Day 17	−25.24	−26.12	−24.86
	−25.36	−26.32	−25.13

Abbreviations: 5βAD, 5β-androstane-3α,17β-diol; 5αAD, 5α-androstane-3α, 17β-diol and PD, pregnanediol
[a]The first collection following testosterone administration.

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is the source of this irreproducibility? Because we know that the reproducibility of $\delta^{13}C^0/00$ measurements of individual steroids in duplicate GC/C/IRMS runs is excellent, we must assume that the *measured* $\delta^{13}C^0/00$ values for individual chromatographic peaks are accurate. If these are accurate, then any difference in the value from the "true" value for the steroid determined must represent some minor contamination of the steroid peak by components with greater or less $\delta^{13}C^0/00$ values. We have proposed that the trend to increasing values for Subject 2 may be because of dietary changes toward foodstuffs with greater ^{13}C content.

These minor irreproducibilities, which typically give rise to a range of about −1.5 in the δ value for all datapoints during the study period, will not affect the outcome of a drug test, because in no cases do the lowest values breach the discriminant value for the positives.

Testosterone/epitestosterone ratios

Table 2 gives the T/E values for five individuals studied here (Subjects 1–5) and three other subjects whose steroids were not analyzed by the IRMS technique (Subjects 6–8). The data show that the maximal increase in the ratio for each of the individuals was between 12 and 68 times baseline level, although it was noticeable that only 4 of the 8 subjects gave T/E values greater than 6 and 1 of these only modestly exceeded it on one occasion (T/E 6.69). The T/E ratio of the five subjects studied here have been included in Figure 5C as compared to the $\delta^{13}C^0/00$ AD/PD ratio for the same individuals.

$\delta^{13}C^0/00$ steroid values for different nationalities

Figure 6 shows the $\delta^{13}C^0/00$ values for duplicate analyses of the androstanediols and PD from 15 individuals of 11 different nationalities in addition to the Chinese. In some cases, it was possible only to obtain values for the 5β androstanediols because of the low amounts of 5αAD present. The results demonstrate that there are differences among racially and regionally varied individuals with undetermined diet, but we must remember that part of this variation may be attributable to the analytical inaccuracies previously discussed. However, all values were greater than −28.2, which should be compared to the values of less than −29.2

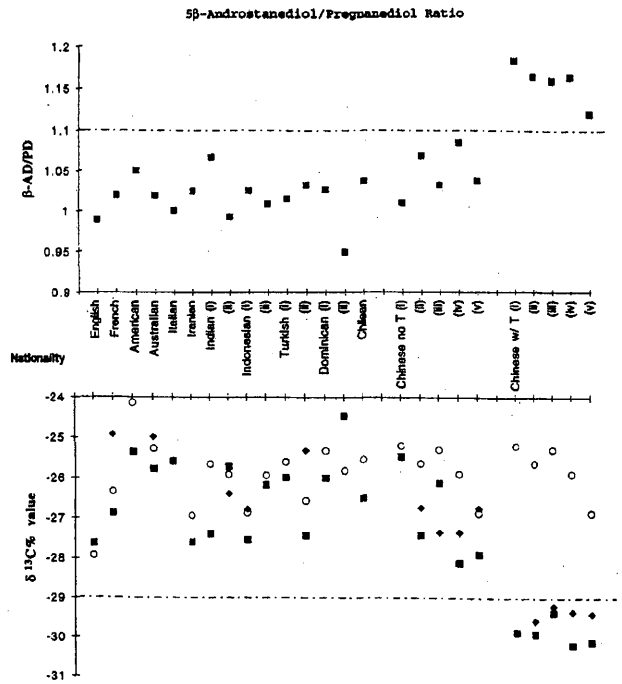

Figure 6 The lower panel shows the $\delta^{13}C^0/00$ values for the androstanediols and pregnanediols for individuals of different nationality, while the upper panel shows the 5βAD/PD ratio. The open circles represent PD, the closed diamonds 5αAD and closed rectangles 5βAD. The baseline values for the Chinese represent the mean of the two measurements, while the Chinese "testosterone" values represent the average of the 7 days after drug administration. Pregnanediol was below detection level in the Chilean urine, so pregnanetriol values were reported instead.

found in the subjects studied following testosterone administration. The mean value for the 5βAD/PD ratio was 1.02 ± 0.03 with a range of 0.95 to 1.07, which compares to the average value for the Chinese baseline samples of 1.05 ± 0.03 (range 1.01 to 1.08). The ratio values for the testosterone administered subjects were all >1.15 in the first days after injection. These data support the previous cut-off ratio value of 1.1:1.0 suggested on the basis of studying the Chinese subjects before and after testosterone administration.

There are notable features with regard to the ^{13}C content

Table 2 Urinary testosterone/epitestosterone ratios for the eight subjects

Study day	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subjec 7	Subject 8
1	0.26	1.28	4.69	0.10	0.12	0.17	0.08	0.11
2	0.33	1.48	5.26	0.11	0.11	0.22	0.06	0.11
3[a]	0.65	8.77	29.3	0.26	0.7	1	1.07	0.42
4	1.95	24.2	36.9	1.77	6.69	4.13	1.36	2.98
5	2.81	45.8	41.8	2.01	5.91	3.53	1.53	7.23
6	2.79	33.2	61.8	1.96	3.71	3.10	1.45	7.48
7	3.03	15.4	54.9	1.91	4.24	3.65	1.68	7.16
8	4.13	36.4	57.7	1.48	5.95	3.08	0.97	4.89
9	5.3	20.3	39.7	1.51	5.78	1.43	1.04	3.81
11	3.2	26.5	33.0	0.58	3.69	0.97	0.40	3.69
13	0.77	2.63	3.42	0.68	3.41	0.20	0.07	1.93
15	0.22	0.99	4.37	0.19	2.27	0.15	0.06	0.21
17	0.26	2.02	4.80	0.13	0.21	0.20	0.08	0.08

[a]Day of testosterone administration.

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of steroids in urine from individuals of different countries. One is that there is a considerable spread in values but one that is unrelated to race and "typical regional diets," at least based on the small sample studied. The lowest and highest $\delta^{13}C^0/00$ among this group differed by 4 units (−24 to −28); however, both these sets of values came from Caucasians. The remaining samples from different nationalities and ethnic groups fell within this range, a particularly important finding with respect to the current study, because it suggests that the results obtained following testosterone administration are universally applicable.

Diet of individuals must have the greatest part to play in the establishment of an individual's $\delta^{13}C$ value. Because all food originates as plant life (including animal protein), it was interesting to research what is known of $\delta^{13}C^0/00$ values for dietary plants. Smith and Epstein[3] published the ^{13}C content of 104 plants, but relatively few were commonly used foodstuffs. A few were high $^{13}C/^{12}C$ plants (corn, sugar cane, grain, and sorghum) averaging around $\delta^{13}C^0/00$ −14, but most were categorized as a separate group of low $\delta^{13}C^0/00$ species having values between −23 and −30. These include dietary oil precursors (olive, sunflower, and castor) and various vegetables (wheat, grass, bamboo, peas, squash, radish, beets, and citrus fruit). Because typical worldwide diets of human and domestic animals use mostly low $\delta^{13}C^0/00$ plants, we would anticipate that $\delta^{13}C$ values for excreted steroids would fall in the −23 to −28 range, as found. The $\delta^{13}C^0/00$ value of an individual plant species does not vary significantly dependent on geographic location,[3] and variation only manifests itself in plants grown in urban areas where CO_2 from fossil fuels increases the ^{12}C content slightly. This clearly does not markedly affect agricultural foodstuffs. For the purposes of this assay technique, it is fortuitous that synthetic testosterone is obviously derived from a very low $\delta^{13}C^0/00$ plant species, otherwise no distinction would be possible between endogenous and exogenous compound.

Practicality of methodology for use in doping control

We compared our $\delta^{13}C^0/00$ values for androstanediol with those of Aguilera et al., who used similar methodologies but different instrumentation.[5] They studied individuals of different nationality and ethnicity than ours. For the purposes of this comparison, we averaged our values for 5α- and 5β-androstanediol, because their values were based on measurement of the combination. For baseline samples, we obtained an averaged $\delta^{13}C^0/00$ of −26.87, which agreed excellently with Aguilera's[5] value of −26.52. For androstanediols measured during testosterone administration, they obtained an average value of −32.44; whereas, our value (the mean of duplicates of the lowest values for each individual) was −30.21. Although our minimum value is not as low as that of Aguilera, it was still well below the baseline level, so it could be used for proving testosterone abuse. The difference in results between the laboratories could be attributed to their use of a testosterone with lower $\delta^{13}C^0/00$ (its origin was not reported) or unequal calibration of the different instruments used.

Our methodology is multistep, albeit built around low-technology procedures. Within the technique, there is only one chromatographic separation, and that was designed to produce a broad fraction rather than the isolation of individual steroids. The key to the method is separate isolation of nonketonic steroids, a procedure that in one extraction step removes 75% of unwanted steroids from urine. A second classical procedure, the oxidative removal of side chains from 17-hydroxypregnane steroids removes many of the remaining complex steroids from the mixture, resulting in a cleaner chromatogram, although in the future, we may simplify the method by discontinuing this procedure if satisfactory results can be obtained. Typically, without using any automation in wet chemistry procedures, a batch of samples requires 1 day of technician time to extract and derivatize. The mass spectrometry was automated, and latterly 20 minutes were required for each run. The maximum number of samples that could be analyzed per 24 h was, therefore, 72.

The length of time that the 250-mg testosterone enanthate dose (equivalent to 180-mg free testosterone) clearly manifested itself in decreased $\delta^{13}C^0/00$ value was 8–10 days. This compares well with other parameters studied on this cohort. For example, plasma testosterone was measured, and from being grossly elevated in the first few days after administration, it returned to normal by the 8th day, although the steroid itself may well be largely of exogenous origin on this occasion.[11] In addition, we report the T/E ratios that are particularly interesting, because essentially only three out of the eight individuals achieve values >6 on more than 1 day, showing a high rate of false negatives. This may not be a universal finding and may well be related to the race (Asian) of the studied individuals. However, it once again emphasizes the importance of developing alternate methodology for proving testosterone misuse. The elevated T/E ratios (although most are <6) fall significantly by the 11th day of the study in all subjects.

We believe that our method based on androstanediol measurement has the needed sensitivity for use in doping control. Although we analyzed all samples in duplicate and plotted the graphs from their averages, individual analyses were equally informative (Figure 4B, C). We would have no hesitation in confirming testosterone administration based on a single measurement. We have been able to get up to five analyses of a single sample using splitless gas chromatographic injection. This represents an equivalent of 5 mL urine for each analysis, which meets sample availability in doping control. Use of a solid injection device for GC, such as glass minivials in a carousel,[12] could improve this sensitivity greatly, because the total sample could be volatized and passed into the GC/IRMS instrument. Testing of a urine volume of 2 mL by our procedure and the Micromass IRMS instrument would be realistic. Our method has two obvious advantages over that of Aguilera et al.[5] One is that the two androstanediols are determined separately, so even if one peak was too small or showed evidence of contamination, the second peak could give the required value. A second advantage is that all analytes are measured in the same chromatogram; whereas, in Aguilera et al.'s study, at least two separate GC/C/IRMS analyses are required to measure the isotope ratio in the androstanediols and their

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ERC cholesterol acetate. However, in fairness, they did have a more difficult assay through the inclusion of testosterone itself among the analytes.

Manipulating the result of a drug test to give a "negative" result following testosterone administration would be difficult. Although the AD/PD ratio could be normalized by taking commercial pregnanediol (Sigma, $\delta^{13}C^0/00$ value −32.12) the low "absolute" value for pregnanediol would give the situation away. Similarly, commercial androstanediols also have very low $\delta^{13}C^0/00$ values (Sigma −34.00) so self-administration of these compounds would not increase the δ values of testosterone derived metabolites. Probably the only way to fool such a test would be to alter the diet drastically over a long period of time through exclusively ingesting foodstuffs with $\delta^{13}C^0/00$ values below −29. False positives could be obtained by ingestion of nonproscribed steroids such as the now commonly used dehydroepiandrosterone (DHEA), which also partially metabolizes to androstanediols. However, whether testosterone or DHEA was being administered could easily be determined from marked differences in the total urinary steroid profile.

Acknowledgments

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JOURNAL OF CHROMATOGRAPHY A

Review

Applied gas chromatography coupled to isotope ratio mass spectrometry

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Abstract

Compound-specific isotope analysis (CSIA) by isotope ratio mass spectrometry (IRMS) following on-line combustion (C) of compounds separated by gas chromatography (GC) is a relatively young analytical method. Due to its ability to measure isotope distribution at natural abundance level with great accuracy and high precision, GC–C–IRMS has increasingly become the method of choice in authenticity control of foodstuffs and determination of origin in archaeology, geochemistry, and environmental chemistry. In combination with stable isotope labelled compounds, GC–C–IRMS is also used more and more in biochemical and biomedical application as it offers a reliable and risk-free alternative to the use of radioactive tracers. The literature on these topics is reviewed from the advent of commercial GC–C–IRMS systems in 1990 up to the beginning of 1998. Demands on sample preparation and quality of GC separation for GC–C–IRMS are discussed also.
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Keywords: Reviews; Food analysis; Mass spectrometry; Isotope analysis; Environmental analysis; Geochemistry; Detection, GC

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1. Introduction

During the 14 years that followed the first publications reporting the coupling of gas chromatography (GC) to on-line combustion of GC separated compounds to yield CO_2 and N_2 for isotope ratio analysis by a single collector mass spectrometer [1,2], hardly any work was published that made use of this new technique. Drawing on this work, in 1984, Barrie et al. [3] coupled a dual collector mass spectrometer to a GC via an on-line combustion interface, thus permitting continuous recording of $(m+1)/m$ isotope ratios by detecting two successive masses at the same time. Their instrument was the first genuine GC–combustion interfaced–isotope ratio mass spectrometry (GC–C–IRMS) system and produced isotope ratios that were an order of magnitude more precise than obtained from an optimised single collector instrument.

However, it was not until 1990 that GC–C–IRMS instruments became commercially available. Since then, GC–C–IRMS instrumentation has experienced several advances and its application has increased to such an extent that it "could almost be considered a conventional technique" [4].

Tom Brenna of Cornell University's Division of Nutritional Sciences, once said "IRMS is probably the first form of analytical mass spectrometry" (see Ref. [166]). IRMS is certainly a very sensitive detector, able to yield highly precise measurements of isotope ratios with a standard deviation in the range of four to six significant figures. When coupled to a GC system, it enables the analyst to conduct highly precise compound-specific isotope analysis (CSIA), especially at natural isotopic abundance level. High-precision CSIA at natural abundance level can provide information on biogenetic relation and origin of a given organic compound. Compared with authentic reference data, subtle differences in the isotopic abundance of ^{2}H, ^{13}C, ^{15}N, or ^{18}O can thus help uncover adulteration of foodstuff or drug abuse in sports to name but a few.

There is also an increasing interest in the application of high-precision CSIA in tracer studies. One area of application is concerned with quantitative studies of biochemical processes such as assimilation/incorporation of nutrients, turnover rates of biologically important molecules, and quantitation of protein synthesis. The other area aims to improve detection limits of bio-organic molecules by using labelled precursor compounds at high enrichment levels.

In either case, high-resolution capillary gas chromatography (HRcGC) is a prerequisite for high-precision CSIA by on-line IRMS. Peak overlap and peak distortion have a detrimental effect on both accuracy and precision of isotope ratio measurements. It is therefore not surprising that scientists working on high-precision CSIA by on-line IRMS invariably employ HRcGC methods.

It is the aim of this article to provide a review over the current spectrum of applied GC–C–IRMS in conjunction with the HRcGC aspects involved. To illustrate as to why the two are inextricably linked, the first section of this review will deal with the characteristics of IRMS and GC–C–IRMS.

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2. Principles of IRMS and GC–C–IRMS

2.1. IRMS

In order to understand why HRcGC is quintessential for high precision CSIA one needs to appreciate exactly how IRMS works. In contrast to so-called organic mass spectrometers (MS) that yield structural information by scanning a mass range (typically over several hundred amu) for characteristic fragment ions, IRMS instruments achieve highly precise measurement of isotopic abundance at the expense of the flexibility of scanning MS.

For isotope ratio measurement, the analyte must be converted into a simple gas, isotopically representative of the original sample, before entering the ion source of an IRMS. Continuous flow isotope ratio measurements of $^{2}H/^{1}H$, $^{15}N/^{14}N$, $^{13}C/^{12}C$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$ are performed on gases of H_2, N_2, CO_2, CO and SO_2, respectively, with ^{13}C abundance measurements accounting for almost 70% of all gas isotope ratio analyses made. One also has to bear in mind that IRMS, in fact, determines the difference in isotope ratio with great precision and accuracy rather than the absolute isotope ratio. IRMS measurements yield the information of isotopic abundance of the analyte gas relative to the measured isotope ratio of a standard or reference gas. This is done to compensate for mass discriminating effects that may fluctuate with time and from instrument to instrument. In dual-inlet IRMS systems, sample gas and standard gas are introduced into two separate gas reservoirs (bellows) and a changeover valve array is used to toggle bellow effluents between the ion source and a waste line, thus maintaining constant viscous flow.

To achieve accurate and highly precise measurement of isotope ratios, obviously great care must be taken to ensure that no part of the analyte data is lost. In the case of CO_2, the data comprise three ion traces for the different isotopomers $^{12}C^{16}O_2$, $^{13}C^{16}O_2$ and $^{12}C^{18}O^{16}O$ with their corresponding masses at m/z 44, 45 and 46, respectively. The three ion beams are registered simultaneously by a multiple Faraday cup (FC) arrangement with a dedicated FC for each isotopomer. The resulting ion currents are continuously monitored, subsequently digitised and transferred to the host computer. Here, the peak area for each isotopomer is integrated quantitatively and the corresponding ratios are calculated.

In both application areas of IRMS (low level enrichment and natural abundance work), small variations in very small amounts of the heavier isotope are detected in the presence of large amounts of the lighter isotope. The abundance A_s of the heavier isotope n_2 in a sample s, given in at.%, is defined as:

$$A_s = R_s/(1 + R_s) \times 100 \text{ (at.\%)} \quad (1)$$

where R_s is the ratio n_2/n_1 of the two isotopes for the sample. The enrichment of an isotope in a sample as compared to a standard value (A_{std}) is given in at.% excess (APE):

$$\text{APE} = A_s - A_{std} \quad (2)$$

Since the small variations of the heavier isotope habitually measured by IRMS are of the order of 0.001–0.05 at.%, the δ-notation in units of per mil (‰) has been adopted to report changes in isotopic abundance as a per mil deviation compared to a designated isotopic standard:

$$\delta_s = (R_s/R_{std} - 1) \times 1000 \text{ (‰)} \quad (3)$$

where R_s is the measured isotope ratio for the sample and R_{std} is the measured isotope ratio for the standard.

2.2. GC–C–IRMS

From the above it is obvious that a GC cannot be directly coupled to an IRMS. The need for sample conversion into simple gases has prompted the design of a combustion interface where the GC effluent is fed into a combustion reactor (Fig. 1). This reactor, either a quartz glass or ceramic tube, is filled with CuO/Pt or CuO/NiO/Pt and maintained at a temperature of approximately 820 or 940°C, respectively [5,6]. The influence of combustion tube packing on analytical performance of GC–C–IRMS has been reported by Eakin et al. [7]. To remove water vapour generated during combustion, a water trap is required. Most instrument manufacturers employ a Nafion tube for this purpose. Nafion is a fluorinated polymer that acts as a semi-permeable

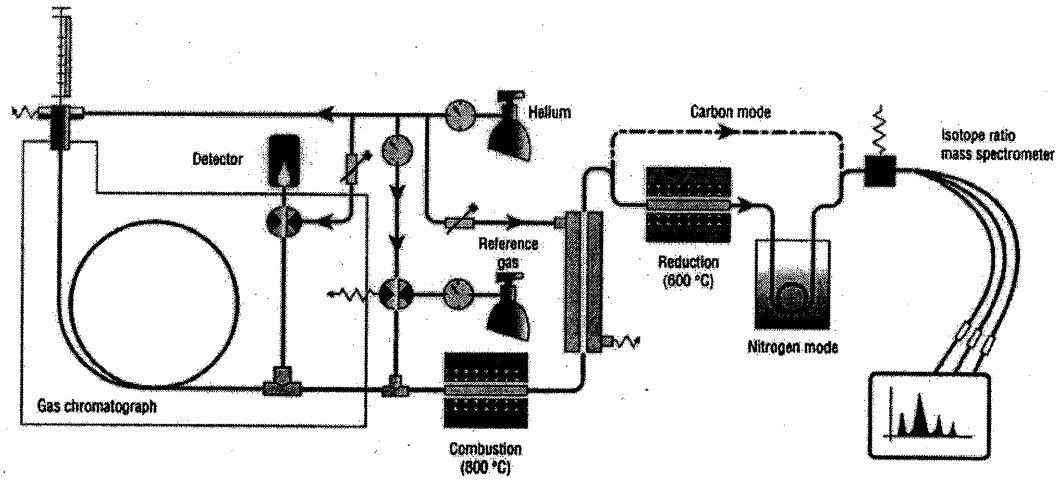

Fig. 1. Set-up of an isotope ratio mass spectrometer coupled to a gas chromatograph via a combustion interface to measure $^{13}C/^{12}C$ (carbon mode) or $^{15}N/^{14}N$ ratios (nitrogen mode). This schematic shows the reference gas set-up used for automated internal isotopic calibration [24].

membrane through which water passes freely while all the other combustion products are retained in the carrier gas stream. Quantitative water removal prior to admitting the combustion gases into the ion source is essential because any water residue would lead to protonation of CO_2 to produce HCO_2^+, which interferes with analysis of $^{13}CO_2$ (isobaric interference). Very recently, a detailed study of this effect has been reported by Lecktrone and Hayes [8].

In dual-inlet systems, the analyte gas comes from a reservoir and only travels a short distance prior to entering the ion source. For this reason, the gas pulses result in rectangularly shaped signals. In contrast, in continuous flow IRMS (CF–IRMS) systems used for gas isotope analysis on-line gas purification steps and overall interface length lead to Gaussian-shaped signals. This is evidently even more pronounced in GC–C–IRMS systems, where analyte peaks eluting from the GC column are fed into an on-line microchemical reactor to produce, e.g., CO_2 peaks. However, due to the chromatographic isotope effect [9–11] the m/z 45 signal ($^{13}CO_2$) precedes the m/z 44 signal ($^{12}CO_2$) by 150 ms on average (Fig. 2) [5], an effect not observed in ordinary CF–IRMS systems. This time displacement depends on the nature of the compound and on chromatographic parameters such as polarity of the stationary phase, column temperature and carrier gas flow [12]. Therefore, loss of peak data due to unsuitably set time windows for peak detection and, hence, partial peak integration will severely compromise the quality of the isotope ratio measurement by GC–C–IRMS, as will traces of peak data from another sample compound due to close proximity resulting in peak overlap with the sample peak to be analysed. Due to the fact that isotope ratios cannot be determined accurately from the partial examination of a GC peak, HRcGC resulting in true baseline separation for adjacent peaks is of paramount importance for high-precision CSIA.

It should be noted, that the chromatographic isotope effect is not caused by a vapour pressure effect but is the result of different solute/stationary phase interactions that are dominated by Van der Waals dispersion forces leading to an earlier elution of the heavier isotopomer [11]. This difference in chromatographic solute/stationary phase interaction is caused by lower molar volumes of the labelled, and thus heavier, compounds. The reason for the decrease in molar volume is the increased bond strength and thus shortened bond length between ^{13}C–H and, to a lesser degree, ^{12}C–^{13}C, and ^{12}C–H and ^{12}C–^{12}C, respectively. If the chromatographic isotope effect would indeed be the result of a vapour pressure effect, one would expect the lighter isotope species of a given compound to elute more rapidly